

CD4⁺ T-Lymphocytes Mediate Ischemia/Reperfusion-induced Inflammatory Responses in Mouse Liver

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Abstract

The success of orthotopic liver transplantation is dependent on multiple factors including MHC tissue compatibility and ischemic/reperfusion injury. Ischemic/reperfusion (I/R) injury in the liver occurs in a biphasic pattern consisting of both acute phase (oxygen free radical mediated) and subacute phase (neutrophil-mediated) damage. Although numerous studies have given insights into the process of neutrophil recruitment after I/R injury to the liver, the exact mechanism that initiates this subacute response remains undefined. Using a T cell-deficient mouse model, we present data that suggests that T-lymphocytes are key mediators of subacute neutrophil inflammatory responses in the liver after ischemia and reperfusion. To this end, using a partial lobar liver ischemia model, we compared the extent of reperfusion injury between immune competent BALB/c and athymic nu/nu mice. Studies evaluating the extent of liver damage as measured by serum transaminases (GPT) demonstrate similar acute (3–6 h) post-I/R responses in these two mouse models. In contrast, the subacute phase (16–20 h) of liver injury, as measured by both serum GPT levels and percent hepatocellular necrosis, was dramatically reduced in T cell-deficient mice as compared with those with an intact immune system. This reduction in liver injury seen in nu/nu mice was associated with a 10-fold reduction in hepatic neutrophil infiltration. Adoptive transfer of T cell-enriched splenocytes from immune competent mice was capable of reconstituting the neutrophil-mediated subacute inflammatory response within T cell-deficient nu/nu mice. Furthermore, in vivo antibody depletion of CD4⁺ T-lymphocytes in immune competent mice resulted in a reduction of subacute phase injury and inflammation as measured by serum GPT levels and neutrophil infiltration. In contrast, depletion of CD8⁺ T-lymphocytes had no effect on these indexes of subacute inflammation. Kinetic analysis of T cell infiltration in the livers of BALB/c mice demonstrated a fivefold increase in the number of hepatic CD4⁺ T-lymphocytes within the first hour of reperfusion with no signifi-

cant change in the number of CD8⁺ T-lymphocytes. In summary, these results implicate CD4⁺ T-lymphocytes as key regulators in initiating I/R-induced inflammatory responses in the liver. Such findings have implications for therapy directed at the early events in this inflammatory cascade that may prove useful in liver transplantation. (*J. Clin. Invest.* 1997. 100:279–289.) Key words: ischemia • reperfusion • liver • T-lymphocytes • CD4

Introduction

Ischemia/reperfusion (I/R)¹-mediated injury has been studied in a number of clinically relevant diseases such as myocardial infarction, cerebrovascular diseases, and peripheral vascular diseases. With the advent of whole organ transplantation, research in the area of I/R injury has been extended to include the commonly transplanted organs kidney, liver, pancreas, and lung. In the liver, I/R injury has been demonstrated to occur in a biphasic pattern: an initial acute phase characterized by hepatocellular damage at 3–6 h and a subacute phase characterized by massive neutrophil infiltration at 18–24 h (1–6). The essential role of neutrophils in the inflammatory phase is supported by studies that demonstrate beneficial effects of neutrophil depletion using CD11b/CD18 monoclonal antibodies (2, 7, 8). These studies also support the notion that neutrophil recruitment and/or endothelial damage may also play an important role in perpetuating hepatic injury after I/R. Although numerous studies have given insight into the process of leukocyte recruitment after I/R injury in the liver, the exact mechanisms that initiate this response remain undefined.

The recruitment of neutrophils after reperfusion likely results from a complex series of ischemia-induced cellular changes in the liver and vasculature that serve to alter the adherent characteristics of neutrophils. Many ischemia-induced changes have been hypothesized to play a role in neutrophil recruitment and the perpetuation of I/R injury including: (a) the release of chemoattractants (such as oxygen free radicals) from ischemically damaged endothelium or hepatocytes (9, 10), (b) Kupffer cell and/or hepatocyte-derived production of inflammatory mediators such as TNF (11, 12), IL-1 (13, 14), and platelet-activating factor (15), (c) cellular changes in microvascular expression of surface antigens such as intercellular adhesion molecule (ICAM) and MHC class II (16), (d) vascular-associated damage creating a “no-reflow” phenomena that may trap neutrophils and further produce a second wave of ischemia (17), and/or (e) the release of superoxides by activated neutrophils that serve to perpetuate chemoattraction of in-

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1. Abbreviations used in this paper: I/R, ischemia/reperfusion; ICAM, intercellular adhesion molecule; Tc, T-cytotoxic; Th, T-helper.

flammatory cell types (9, 10). Numerous studies suggest that oxygen free radical formation after reoxygenation of the liver may initiate the cascade of hepatocellular injury, necrosis, and subsequent inflammatory infiltration (17–20). For example, studies aimed at reducing reactive oxygen species after I/R injury, with xanthine oxidase inhibitor allopurinol and exogenous free radical scavengers such as SOD and catalase, have demonstrated beneficial effect (21–25). However, the source and mechanism of these free radicals remains controversial and may involve Kupffer cells, hepatocytes, extracellular xanthine oxidase in the acute phase, and neutrophils at the inflammatory stage (26–29).

The ambiguity in the field as to the mechanisms of I/R-induced liver injury results from a lack of understanding of the pathophysiologically relevant primary inciting events that lead to the subacute inflammatory responses and the ultimate decline of liver function. T-lymphocytes have been demonstrated to be key cellular mediators of granulocytic inflammatory responses through several secreted cytokines including TNF α , IFN γ , IL-4, and GM-CSF (30). These cytokines regulate proliferation, accumulation, and attraction of granulocytes to the site of injury. T cells are divided into three categories including T-helper (Th), T-cytotoxic (Tc), and T-suppressor (Ts) cells. Th cells are in turn subdivided into two categories based on their functional pattern of secreted cytokines; Th1 cells secrete IL-2 and IFN γ , but relatively little IL-4 or IL-5, whereas Th2 cells secrete IL-4 and IL-5, but little IL-2 and IFN γ (30). To better define the mechanisms of leukocyte recruitment in the liver after I/R, we sought to investigate the involvement of T-lymphocytes in this recruitment process. We reasoned that by comparing I/R responses in the liver between nu/nu and syngeneic immune competent mice, we would be able to study those responses that are specifically due to T cell functions. To this end, we compared pathophysiologic endpoints of hepatocellular and immune responses after lobar-induced I/R injury in the liver between nu/nu and BALB/c mice. These studies implicate T cell lineages in the progression of acute I/R injury to subacute inflammatory stages of leukocyte recruitment.

Methods

Mouse model of lobar I/R injury. Two mouse models including BALB/c and nu/nu mice (Harlan Sprague-Dawley Inc., Indianapolis, IN) were evaluated for I/R injury responses in the liver. Male (25 g) mice were anesthetized with ketamine/xylazine and a laparotomy was performed to expose the liver. After surgical exposure of the liver, mice were injected with heparin (100 μ g/kg) to prevent clotting of blood at long time points of ischemia. The medial largest lobe of the liver was clamped at its base using a microaneurysm clamp followed by placement of the liver and clamp back into the peritoneal cavity for 3, 60, or 90 min. Sham surgeries were performed on anesthetized and heparinized animals for which the liver was not clamped. 3-min ischemic time points were performed to control for any nonspecific hepatocellular damage induced by clamping, which was unassociated with long periods of ischemia. All animals remained anesthetized for a total of 90 min to control for any effects of anesthesia. After surgically implemented ischemia, the abdominal wall was sutured with two layers and the animals were returned to their cages. A total of four serial 12–25 μ l retro-orbital bleeds were performed on each animal at various time points including 0, 3, 6, 12, 16, 20, and 36 h postreperfusion, and serum was collected immediately by centrifugation and stored on ice. Although the number of bleeds for any one animal was limited to four, all animals were bled at 0 h to obtain an accurate baseline. After

completion of the experiment, serum GPT levels were measured using a microkinetic assay (200 μ l total volume; Sigma Chemical Co., St. Louis, MO). Typically assays were performed with 6 μ l of serum for preischemic and 2 μ l for postischemic time points. Assays were based on a variation of Sigma Chemical Co. kits to accommodate the small volumes of serum in which the change in OD_{340nm} was assessed in a 96-well microtiter plate reader (Molecular Dynamics, Sunnyvale, CA). Serum standards were used to assess the linearity of the assay and to convert values to international units per liter GPT. In total, 6–10 animals were evaluated at each time point of ischemia, and enzyme assays were performed in duplicate. Livers were harvested after the 20- or 36-h bleeds and divided into ischemic and nonischemic lobes. All tissues were divided into regionally identical quadrants that were either frozen directly in ornithine carbamyl transferase or fixed in formalin. The extent of liver damage as indicated by hepatocellular necrosis was confirmed by blinded morphometric assessment in paraffin sections (4 μ m) using a Leica DMR image analysis system to determine the percent necrotic area in liver sections. In these studies, the area of necrosis was traced by computer-assisted morphometry and the percentage of necrosis calculated from the total micrometer² area of the tissue section. In total, four sections were analyzed for each ischemic lobe tissue sample ($n = 4$ independent animals for each experimental point). Nonischemic lobes, which demonstrated no significant necrosis, except for infrequent focal areas at the surface of the liver due to surgical manipulation, were not included in this analysis. All statistical comparisons were performed using the Student's t test with significance inferred by $P < 0.05$.

Purification of T cell-enriched populations from splenocytes. Mononuclear cell suspensions were generated from spleens of immune competent BALB/c mice. Freshly isolated spleens were placed in complete RPMI (Hepes) media containing 10% FCS and cells removed by blunt dissection and scraping through a metal mesh. Cell aggregates were further separated by filtration through a 200- μ m mesh nylon screen. Splenic cells were collected by centrifugation and the red blood cells were removed by hypertonic lysis in NH₄Cl for 5 min (31). Mononuclear cells were harvested by centrifugation and washed three times in 10% FCS containing media. T cell enrichment was performed using nylon wool column chromatography as previously described (31). Briefly, sterile nylon wool (Fisher Scientific Co., Santa Clara, CA) packed columns were equilibrated in complete RPMI media and mononuclear cell fractions incubated on the column for 1 h at 37°C in 5% CO₂. Nonadherent cells (predominately T cells) were then eluted in 15 ml complete media and collected by centrifugation. The percentages of CD4⁺ and CD8⁺ T cells were assessed in nylon wool T cell-enriched fractions by flow cytometry. In these studies, 5×10^5 cells from the nylon wool column were washed once with 5% FCS/PBS and resuspended in the same buffer containing 1 μ g of PE anti-mouse CD4 and FITC anti-mouse CD8 antibodies (PharMingen, San Diego, CA) for 2 h at 4°C. Stained cells were washed three times in 5% FBS/PBS, analyzed by flow cytometry.

Adoptive transfer of T cells into nu/nu mice. T cell-enriched splenocyte fractions composed of $> 80\%$ T cells as indicated by CD4 and CD8 FACS[®] analysis (Becton Dickinson & Co., Mountain View, CA) were injected into nu/nu mice by subcapsular splenic injection 5 d before I/R experiments. Two experimental protocols were performed in which a total of either 2×10^5 or 2×10^6 cells resuspended in PBS were injected in each animal. Because the lower dose of T cells gave no experimental response, the data presented in this report represents only that from the higher dose ($n = 6$). All animals were subjected to 60 min of lobar ischemia followed by reperfusion. Blood was drawn at 0, 6, and 20 h post-I/R and analyzed for serum GPT levels. The extent of subacute I/R injury was assessed by comparing the extent of decline in serum GPT responses at 6 h to that at 20 h post-I/R.

Immunocytochemical localization of neutrophils and T cells. T cell localization studies were performed by immunofluorescent staining for CD4 and CD8 antigens in frozen (6- μ m) sections of the liver and spleen. In these studies, sections were fixed in 95% ethanol for 30 min, blocked in 20% mouse serum/PBS for 30 min, and then incubated in

1/200 dilution of rat FITC anti-mouse CD4 or rat FITC anti-mouse CD8 in the presence of 1.5% mouse serum/PBS for 18 h at 4°C. Sections were washed three times in 1.5% goat serum/PBS and coverslipped in Citifluor. These analyses were performed to assess the kinetics of T cell infiltration to the liver post-I/R and to confirm the success of T cell adoptive transfers. Kinetic T cell infiltration studies assessed ischemic lobes of livers from mice that underwent 90 min of ischemia and 1, 3, 6, 12, and 20 h of reperfusion (three independent animals were analyzed for each experimental group). Additionally, sham-operated animals were included in these analyses to assess baseline numbers of resident T-lymphocytes in the liver.

Immunofluorescent staining for neutrophils was performed on frozen sections fixed in 4% paraformaldehyde for 10 min, followed by postfixation in methanol for 10 min at -20°C. Sections were permeabilized with 0.1% Triton X-100 before blocking and incubation with a 1/500 dilution of rat anti-mouse neutrophil FITC antibody (CALTAG Labs, South San Francisco, CA). Morphometric analysis of immunoreactive neutrophils in liver sections were performed by quantifying the total numbers of immunoreactive cells in 30 random (400×) fields (five independent animals were analyzed for each experimental group). Localization of neutrophils in CD4 and CD8 T cell-depleted animals assessed 10 random fields (400×) of the ischemic liver lobes from five and three independent animals, respectively.

In vivo T cell depletion. Rat monoclonal antibodies against mouse CD4 and CD8 were generated by intraperitoneal injection of 10⁷ cells of the rat hybridoma lines GK 1.5 or 2.43, obtained from American Type Culture Collection (Rockville, MD) (32, 33), into female SCID mice. Ascites fluid was harvested and purified over an anti-rat IgG agarose (Sigma Chemical Co.) column. Positive fractions were identified by Bradford analysis and purity verified by SDS-PAGE. The positive fractions were then pooled, desalted, and concentrated into PBS using a Centricon-30 filtration system. Antibody concentrations were adjusted to 1 mg/ml. The mAb were then tested by flow cytometry analysis for antigen specificity by comparison with commercially available directly labeled anti-CD4 and -CD8 antibodies. In vivo depletion experiments were performed in 6–7-wk-old BALB/c mice by injection of 0.2 mg of affinity-purified anti-CD4 mAb (GK1.5) or anti-CD8 mAb (2.43) on two consecutive days. On day 4 after the second injection, the animals underwent 90 min of ischemia as described above and blood samples were taken at 0, 6, 12, and 20 h post-reperfusion for analysis of serum GPT. The animals were then killed and spleens from each mouse were tested for successful T cell depletion by flow cytometry analysis. In these studies, total splenocytes were isolated and purified as described above and 10⁶ cells stained with 1 µg of anti-CD8 FITC- and anti-CD4 PE-labeled antibodies in 50 µl PBS/10% FCS for 30 min on ice.

Results

Reduced subacute I/R injury response in livers from nu/nu mice. Results from both 60- and 90-min lobar liver I/R injury in both BALB/c and nu/nu mice demonstrate similar acute (3–6 h) increases (50–100-fold) in the level of serum GPT (Fig. 1, A and B) over preischemic time points. Conversely, 3-min ischemic animals showed minimal (threefold) transient increases in GPT at 3- and 6-h time points, which were likely due to nonspecific hepatocellular clamping damage. Sham-operated animals produced no detectable changes in serum transaminases, demonstrating that neither anesthesia nor serial blood draws affected these liver function enzymes. In contrast with the similar level of acute I/R-mediated liver injury seen in BALB/c and nu/nu mice, subacute (20-h) levels of serum GPT were dramatically different between these two strains. Serum GPT values of BALB/c animals that underwent 90 min ischemia demonstrated a biphasic pattern of liver injury that peaked at 6 and 20 h, representing early acute and subacute

damage, respectively. In comparison, nu/nu mice after 90 min of ischemia demonstrated a complete lack of subacute-mediated liver injury, suggesting that the inflammatory response after I/R injury in T cell-deficient mice was impaired (compare Fig. 1 A to B). Although the bimodal serum GPT phases of liver injury were not observed after 60 min of I/R, similar differences in the decline of serum GPT levels were seen during the later phases of I/R between nu/nu and BALB/c mice. At 36 h, the GPT activities for all animals returned to baseline. Morphometric analysis of the percentage of hepatocellular necrosis confirmed differences in the level of hepatic injury at 20 h post-reperfusion in BALB/c and nu/nu mice (Fig. 1, C–I). BALB/c animals subject to 60 and 90 min of ischemia demonstrated a twofold higher level of hepatocellular necrosis as compared with nu/nu mice ($P < 0.009$ and $P < 0.001$ for 60 and 90 min ischemia, respectively). In contrast, control animals subject to 3 min of ischemia demonstrated negligible levels of necrosis in both strains of mice. Differences in the extent of the bimodal serum GPT response between BALB/c animals, which had undergone 60- and 90-min of I/R injury, is likely due to differences in the extent of injury produced by these two times of ischemia and is reflected in the percent hepatocellular necrosis ($27 \pm 4.8\%$ for 60 min of ischemia vs. $62 \pm 9.0\%$ for 90 min) (Fig. 1 J). In summary, initial results comparing the time course and extent of I/R injury in the liver between nu/nu and BALB/c mice demonstrate similar patterns of acute I/R injury, but dramatic differences in the extent of subacute responses. Such findings suggest that the absence of T cells in nu/nu mice may be responsible for reduced subacute injury responses after I/R. To address this hypothesis, we attempted to reconstitute this subacute I/R response in nu/nu mice by adoptive transfer of T-lymphocytes harvested from BALB/c mice.

Adoptive transfer of T cells reconstitutes the subacute inflammatory response in nu/nu mice. Enriched populations of T cells were purified from BALB/c splenocytes using nylon wool chromatography. The extent of T cell enrichment was confirmed by flow cytometry after immunocytochemical staining with CD4 and CD8 antibodies (Fig. 2 A). These results demonstrated an 80% T cell content within glass wool-purified splenocytes. Within these T cell populations, ~ 25 and 55% were positive for CD8 and CD4, respectively. Adoptive transfer into nu/nu mice was performed by injection of 2×10^6 enriched T cells directly into the subcapsular region of the spleen. The successful engraftment of T cells was evaluated by immunocytochemical staining of spleens (Fig. 2) for both CD4 and CD8 antigens (epitopes that are normally absent in nu/nu mice). In these studies, nu/nu animals demonstrated no detectable CD4 and CD8 T cells within the spleen (Fig. 2, E and F) as compared with control BALB/c mice (Fig. 2, C and D). In contrast, nu/nu mice injected with enriched T-lymphocyte populations (Fig. 2, G and H) demonstrated high levels of CD4 and CD8 immunoreactive cells in the spleen. These results confirm the successful reconstitution of T-lymphocytes over the time course of these experiments.

The profile of serum GPT levels was quantified in animals after 60 min I/R in an attempt to evaluate whether adoptively transferred T cells were capable of reconstituting the subacute injury response. Results from these studies demonstrated a fourfold increase in 20-h GPT levels ($P < 0.001$) within T cell adoptively transferred nu/nu mice ($2,029 \pm 317$ U/liter) as compared with uninjected nu/nu animals (499 ± 83 U/liter). In nu/nu mice that received T cells, the extent of the subacute injury

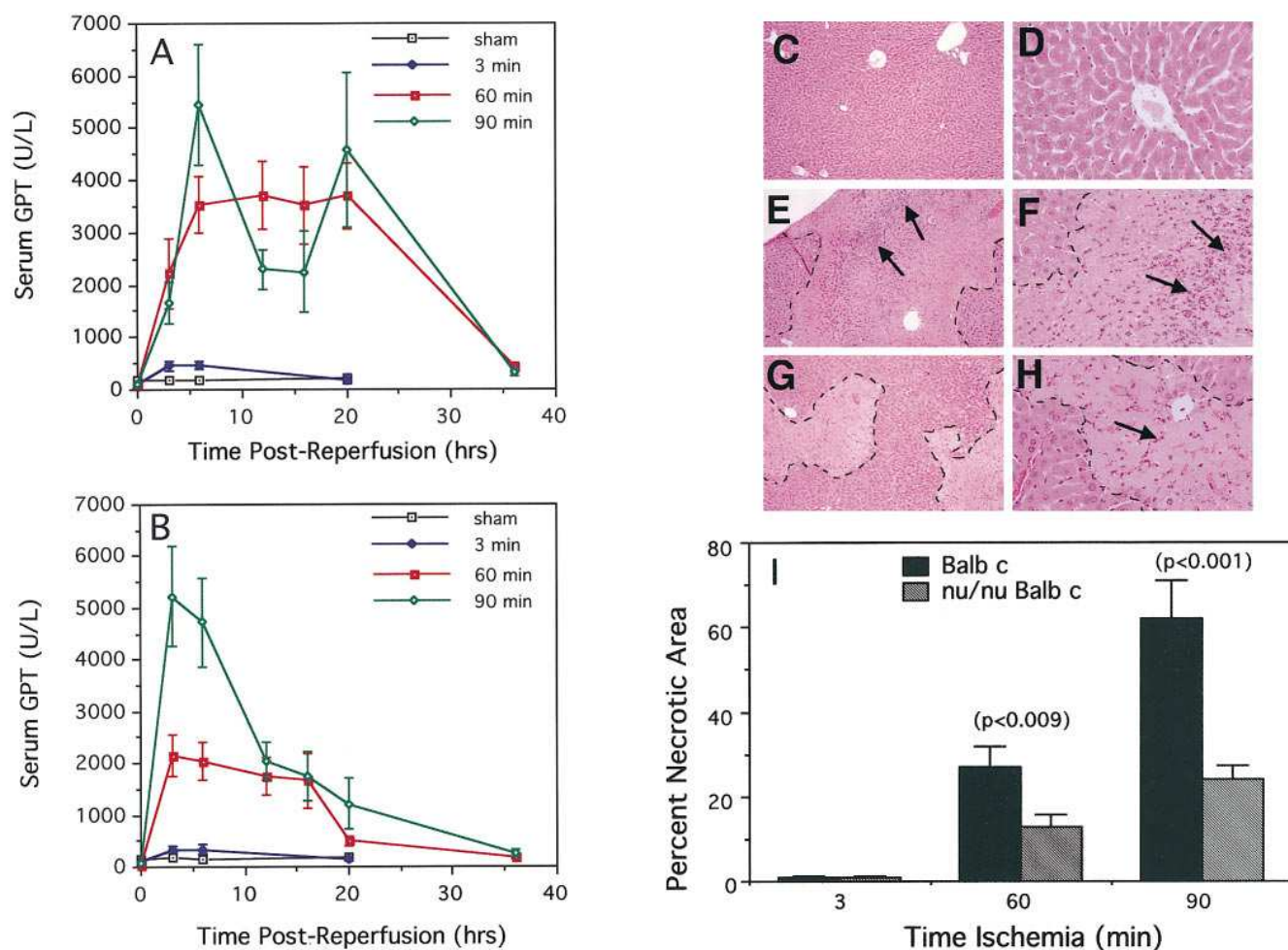


Figure 1. Differences in I/R-induced liver damage in BALB/c and nu/nu mice. BALB/c (A) and nu/nu (B) mice were subject to lobar ischemia for 3, 60, and 90 min, followed by reperfusion for a total of 20–36 h. Sham animals for which surgery was performed without ischemia were included as additional controls. Blood samples from animals were analyzed for serum GPT levels at 0, 3, 6, 12, 16, 20, and 36 h postreperfusion. For any one animal, only four serial blood draws were performed in an effort to assure minimal changes in blood volume. In 60- and 90-min ischemia experiments, the total number of independent animals for each time point were as follows: 0 ($n = 15$), 3 ($n = 6$), 6 ($n = 10$), 12 ($n = 9$), 16 ($n = 9$), 20 ($n = 10$), and 36 ($n = 6$) h. For all sham and 3-min ischemia experiments, the total number of animals for each time point presented was $n = 6$. A (BALB/c) and B (nu/nu) represent the mean \pm SEM of serum GPT values of pooled data for each group. Ischemic and nonischemic lobes were harvested for morphologic analysis of the percent hepatocellular necrosis in paraffin sections. Photomicrographs from sections of 3-min (C and D) and 60-min (E and F) BALB/c ischemic lobes are compared with ischemic lobes from nu/nu mice after 60 min of ischemia (G and H). All livers were harvested at 20 h postreperfusion for evaluation of percent hepatocellular necrosis. Panels on the left and right were taken with 4 \times and 40 \times objectives, respectively. Dashed lines outline the boundaries of necrosis while arrows point to inflammatory infiltrate. The mean percent area \pm SEM of hepatocellular necrosis is shown in I for ischemic lobes ($n = 4$ independent animals for each experimental point). Percent hepatocellular necrosis was significantly higher in BALB/c animals subject to both 60 (Student's t test, $P < 0.009$) and 90 (Student's t test, $P < 0.001$) min of ischemia as compared with nu/nu animals. No significant differences were seen in the percent necrosis between BALB/c and nu/nu livers subject to 3 min of ischemia. Nonischemic lobes did not demonstrate necrosis at any of the time points of ischemia (data not shown).

was increased to a level $\sim 50\%$ that seen in BALB/c mice ($3,704 \pm 625$ U/liter). Fig. 2 B represents the cumulative data from these experiments, which compared the peak acute (6 h) GPT levels with peak subacute (20 h) serum GPT levels. Studies evaluating T cell recruitment to the liver after I/R injury in adoptive transfer experiments provided additional evidence supporting the importance of T cells in subacute phase liver damage. Nu/nu mice adoptively transferred with purified splenic T cell populations demonstrated T cell infiltration into the liver after I/R. In all cases, T cell infiltration was confined to the ischemic lobes of the liver and absent in control nonis-

chemic lobes (data not shown). These findings demonstrate that the elevated levels of CD4 $^{+}$ T cells in the livers of adoptively transferred nu/nu mice were not due to a nonspecific global increase in resident T cell in the liver, but rather a specific response to I/R injury. As seen with BALB/c mice, no significant infiltration of CD8 $^{+}$ T cells were seen in nu/nu mice adoptively transferred with purified splenic T cell populations (data not shown).

In summary, these findings demonstrate that adoptively transferred T cells were capable of reconstituting the subacute I/R injury response in nu/nu mice and suggested that T cells

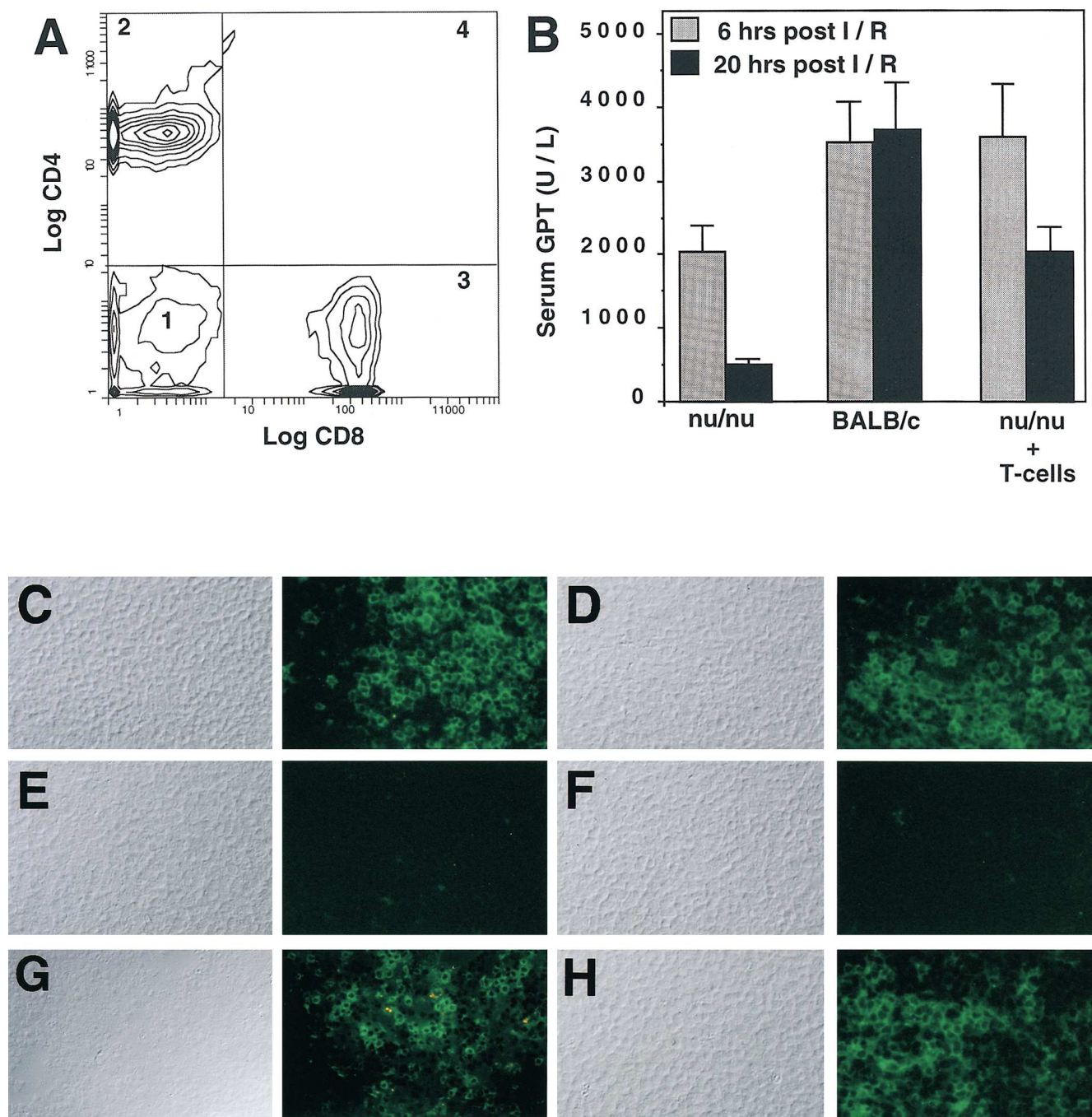


Figure 2. Adoptive transfer of T-lymphocytes into nu/nu mice. Enriched populations of T-lymphocytes were isolated from spleens of BALB/c mice by nylon wool chromatography and analyzed by flow cytometry after immunocytochemical staining with anti-mouse CD4 and CD8 antibodies (A). Results from FACS[®] analysis demonstrate an enrichment to > 80% T cells. Within these T cell fractions, ~ 25% (quadrant 3) and 55% (quadrant 2) of the splenocytes were positive for CD8 and CD4, respectively. 2×10^6 T-lymphocytes were injected into the splenic subcapsular space of recipient nu/nu mice ($n = 6$ for each experimental group). 5 d posttransfer, mice were subject to 60 min of partial lobar ischemia followed by 20 h of reperfusion. B compares serum GPT levels observed during the peak acute (6 h) and subacute (20 h) phases of post-I/R injury between nu/nu, Balb/c, and nu/nu T cell-transplanted mice. Spleens were harvested at 20 h postreperfusion to determine the efficiency of adoptive transfer by immunocytochemical analysis with CD4 and CD8 antibodies. Immunocytochemical staining of spleen-frozen sections (6 μ m) with anti-mouse CD8 FITC (C, E, and G)– and anti-mouse CD4 FITC (D, F, and H)–labeled antibodies was performed on BALB/c mice (C and D), nu/nu mice, which were uninjected (E and F), or nu/nu mice injected with T cell populations into the spleen (G and H). BALB/c and nu/nu mice serve as positive and negative controls for CD4 and CD8 staining, respectively. All panels represent photomicrographs taken with a 20 \times objective. Nomarski and fluorescent photomicrographs are shown on the left and right of each panel, respectively.

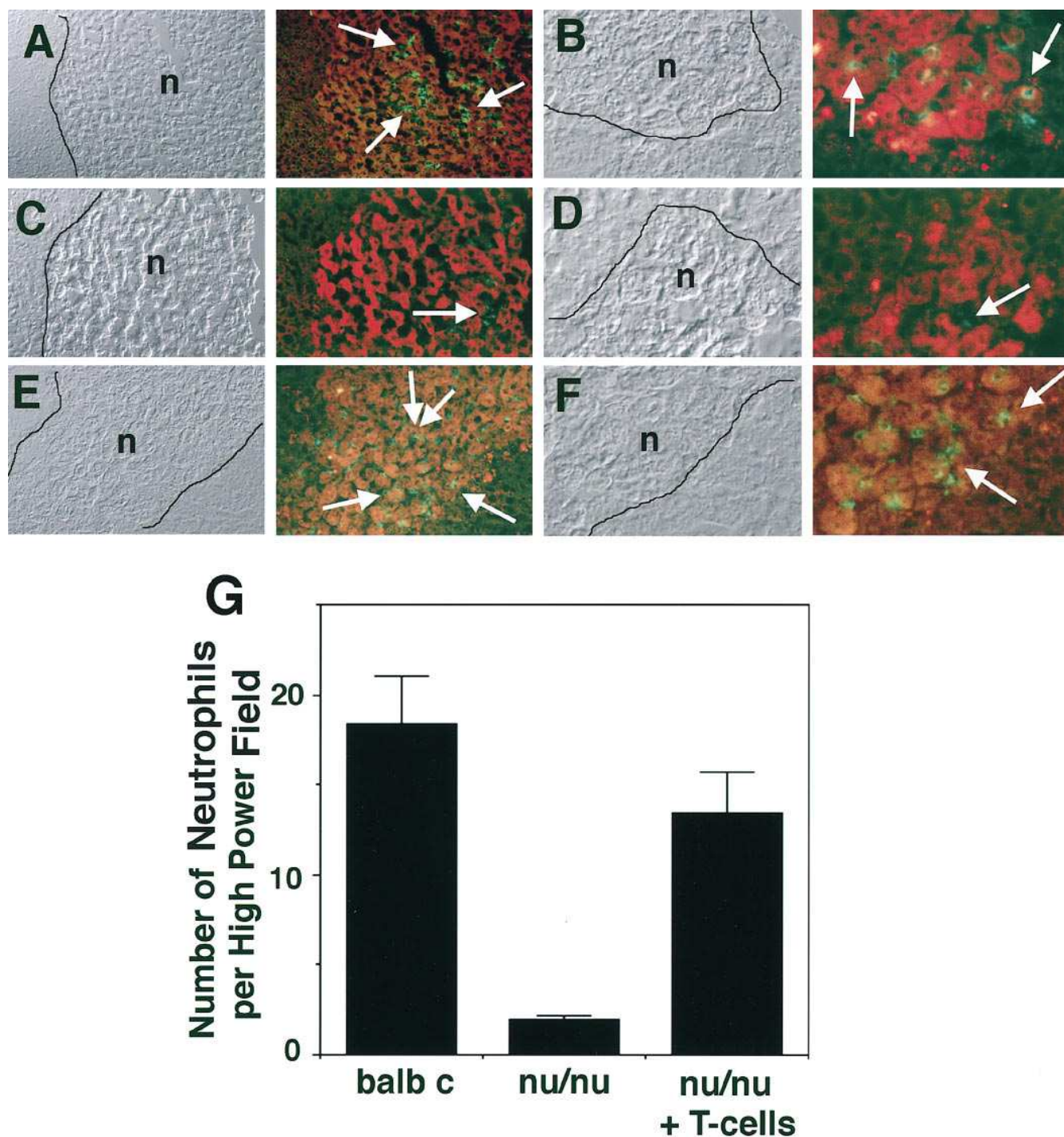


Figure 3. Immunocytochemical localization of neutrophils within T cell-defined animal models of I/R in the liver. Frozen sections ($6\ \mu\text{m}$) of ischemic livers were immunocytochemically stained for neutrophils using an anti-mouse neutrophil FITC-labeled antibody. Three experimental groups were compared for the extent of neutrophil infiltration within 60-min ischemic lobes at 20 h of reperfusion (peak neutrophil response). Immunoreactive cells were quantified from 30 fields within necrotic regions of ischemic lobes ($n = 5$ independent animals for each group). Nonischemic lobes were also analyzed but did not demonstrate detectable neutrophil infiltration (data not shown). Representative immunofluorescent photomicrographs are shown for ischemic lobes from BALB/c (*A* and *B*), nu/nu (*C* and *D*), and T cell-transplanted nu/nu mice (*E* and *F*). Nomarski and fluorescent photomicrographs are shown on the left and right of each panel, respectively. *A*, *C*, and *E* represent photomicrographs taken with a $10\times$ objective, while *B*, *D*, and *F* were taken at higher magnification with a $40\times$ objective. Lines within the Nomarski photomicrographs mark the boundaries of necrotic regions that demonstrate a red/orange autofluorescence using this Texas red/FITC filter cube. Arrows mark immunoreactive neutrophils with green FITC fluorescence. A summary of the quantitative analysis is depicted in *G* that gives the mean \pm SEM number of immunoreactive neutrophils per high power field ($40\times$). The number of neutrophils in the ischemic liver lobes were significantly reduced in nu/nu mice as compared with BALB/c or nu/nu T cell transplanted mice (Student's *t* test, $P < 0.001$).

may be critical mediators in this phase of injury, which has been traditionally characterized as a neutrophil-mediated inflammatory response. To this end, we sought to correlate the requirement of T cells necessary for subacute phase I/R injury with the extent of neutrophil infiltration.

T cells mediate neutrophil recruitment after ischemia/reperfusion in the liver. Subacute injury after I/R has been traditionally characterized by massive neutrophil-mediated inflammatory responses. Numerous hypotheses have proposed mechanisms by which ischemic injury in the liver may mediate neutrophil recruitment, including hepatocellular cytokine production and local changes in the adherence characteristic of endothelium in the liver. Initial comparisons of I/R histopathology between nu/nu and BALB/c mice shown in Fig. 1, E–H have suggested that neutrophil recruitment may be impaired in T cell-deficient mice. These findings, together with the requirement for T cells to elicit I/R subacute liver injury, have

suggested that T-lymphocytes may be key mediators of this response. To this end, we sought to correlate differences in the level of subacute injury with the extent of the subacute neutrophil inflammatory response in nu/nu and BALB/c mice. Furthermore, by comparing nu/nu with BALB/c and T cell-reconstituted nu/nu mice, we sought to mechanistically confirm T cell involvement with the activation of neutrophil infiltration.

Immunocytochemical localization using a pan antineutrophil antibody was performed on five to six animals from each experimental category that had undergone 60 min of ischemia followed by 20 h of reperfusion. This time point was chosen for analysis since subacute liver injury peaks during this time frame. Morphometric studies quantifying the extent of neutrophil infiltration in the liver demonstrated a mean number of 18.4 ± 2.6 immunoreactive neutrophils per high power field in necrotic regions of BALB/c ischemic lobes (Fig. 3, A and B). Only infrequent immunoreactive cells were seen in nonne-

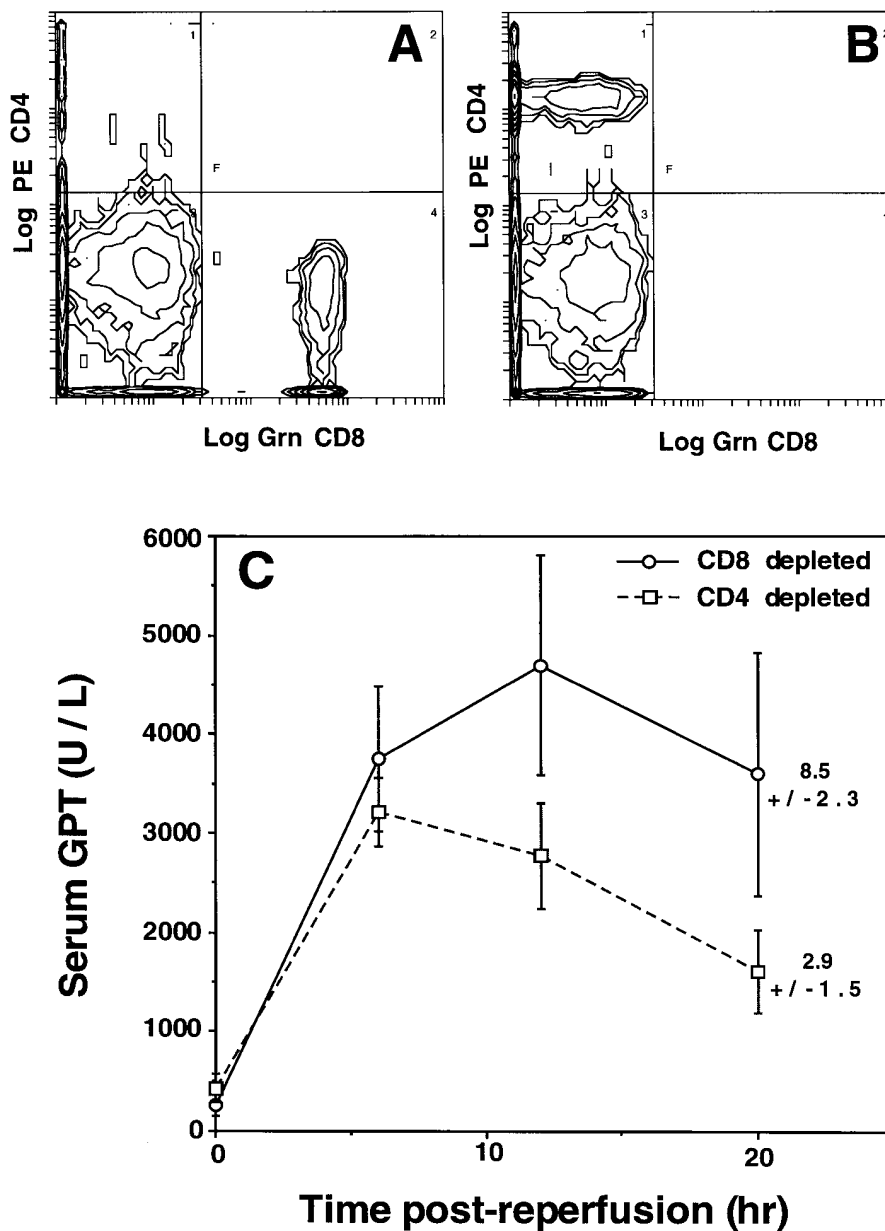


Figure 4. Modulation of I/R injury responses after in vivo depletion of CD4⁺ and CD8⁺ T-lymphocytes in BALB/c mice. 6–7-wk-old BALB/c mice were depleted of CD4⁺ ($n = 7$) or CD8⁺ ($n = 4$) T-lymphocytes by injection of affinity-purified anti-CD4 or anti-CD8 mAb on two consecutive days. 4 d after the second injection, animals underwent 90 min of partial lobar ischemia and blood samples were taken at 0, 6, 12, and 20 h postreperfusion for analysis of serum GPT. Animals were killed at 20 h post-I/R and the spleens from each mouse were tested for successful T cell depletion by FACS[®] analysis against anti-CD8 FITC- and anti-CD4 PE-labeled antibodies. The antibodies used for FACS[®] analysis recognized different epitopes from those used for in vivo depletion. FACS[®] analysis of splenocytes from CD4- (A) and CD8- (B) depleted animals are shown. The mean \pm SEM serum GPT profile for both CD4- (dashed line) and CD8- (solid line) depleted mice are given in C. GPT levels were significantly lower in CD4- as compared with CD8-depleted animals at both 12 and 20 h of reperfusion (Student's *t* test, $P < 0.05$). The mean \pm SEM number of neutrophils per 40 \times field within ischemic lobes at 20 h postreperfusion are given for both CD4- and CD8-depleted animals (shown to the right of 20-h GPT experimental values). These data demonstrate a statistically significant decrease in the number of neutrophils within ischemic lobes of CD4- as compared with CD8-depleted animals (Student's *t* test, $P < 0.05$). Quantification of neutrophil numbers was performed by immunofluorescent detection as described in the Methods.

crotic regions of ischemic lobes, while neutrophils were absent from control nonischemic lobes (data not shown). In contrast with BALB/c animals, nu/nu mice demonstrated a 10-fold reduction (1.9 ± 0.3 cells/field, $P < 0.001$) in the number of neutrophils within necrotic regions of ischemic lobes (Fig. 3, C and D). Such findings suggest that the absence of T cells in nu/nu mice may be responsible for the reduced subacute neutrophil inflammatory response seen at 20 h postreperfusion. To conclusively address this hypothesis, nu/nu mice that were adoptively transferred with an enriched population of splenic T cells were analyzed for neutrophil infiltration after I/R. T cell-transplanted mice subjected to 60 min of lobar ischemia followed by 20 h of reperfusion demonstrated a statistically significant ($P < 0.001$) sevenfold increase in the number of immunoreactive neutrophils within ischemic lobes (13.4 ± 2.3 cells/field) (Fig. 3, E and F). Control nonischemic lobes from these animals demonstrated an absence of neutrophils as similarly seen in nu/nu and BALB/c mice (data not shown), suggesting that adoptive transfer of T cells did not globally elevate circulating neutrophils in the liver, but rather specifically increased the neutrophil inflammatory response to I/R. Morphometric analysis of I/R-induced increases in the frequency of neutrophils seen in necrotic regions of the liver are summarized in Fig. 3 G. When the ratio of 20-h/6-h serum GPT levels (an index of subacute mediated injury) were grouped for all animals, including nu/nu, Balb/c, and nu/nu + T cells, and compared with the extent of neutrophil infiltration, a significant direct correlation ($r = 0.9$, $P < 0.001$ by the Spearman correlation) was observed in the frequency of neutrophil infiltration and the extent of subacute serum GPT levels. Such find-

ings substantiate the hypothesis that T-lymphocytes play a key role in the recruitment of neutrophils in subacute I/R injury.

Depletion of CD4⁺ but not CD8⁺ T cells decreases the subacute inflammatory response in BALB/c mice. To determine whether specific subsets of T cells were responsible for activating subacute inflammatory responses in the liver seen at 18–24 h after reperfusion, we selectively in vivo-depleted CD4⁺ and CD8⁺ T cells from BALB/c mice before I/R experiments. To this end, animals were injected intraperitoneally with depleting monoclonal antibodies derived from rat hybridomas directed against the CD4 or CD8 receptors. The extent of depletion was confirmed in all animals after I/R experiments by FACS[®] analysis of splenocytes with CD4 and CD8 antibodies that recognized alternative epitopes to antibodies used for depletion studies. All mice demonstrated > 95% depletion of immunoreactive CD4 or CD8 cells after depletion experiments (Fig. 4, A and B). CD4- and CD8-depleted animals were subjected to 90 min of partial lobar ischemia, and the extent of liver injury was monitored by measuring serum GPT values at 0, 6, 12, and 20 h. While there were no significant differences in baseline (0 h) and 6 h reperfusion GPT values among the different groups of animals, the GPT activities at 12 and 20 h were significantly lower ($P < 0.05$) in CD4-depleted mice as compared with the CD8-depleted group (Fig. 4 C). CD4-depleted animals demonstrated a similar pattern of GPT injury as seen in nu/nu (T cell-deficient) mice, while CD8-depleted animals more closely reproduced the pattern of injury seen in BALB/c (T cell-sufficient) mice. Furthermore, CD4-depleted BALB/c had significantly reduced ($P < 0.05$) numbers of neutrophils (2.9 ± 1.5 cells/field) as compared with CD8-depleted

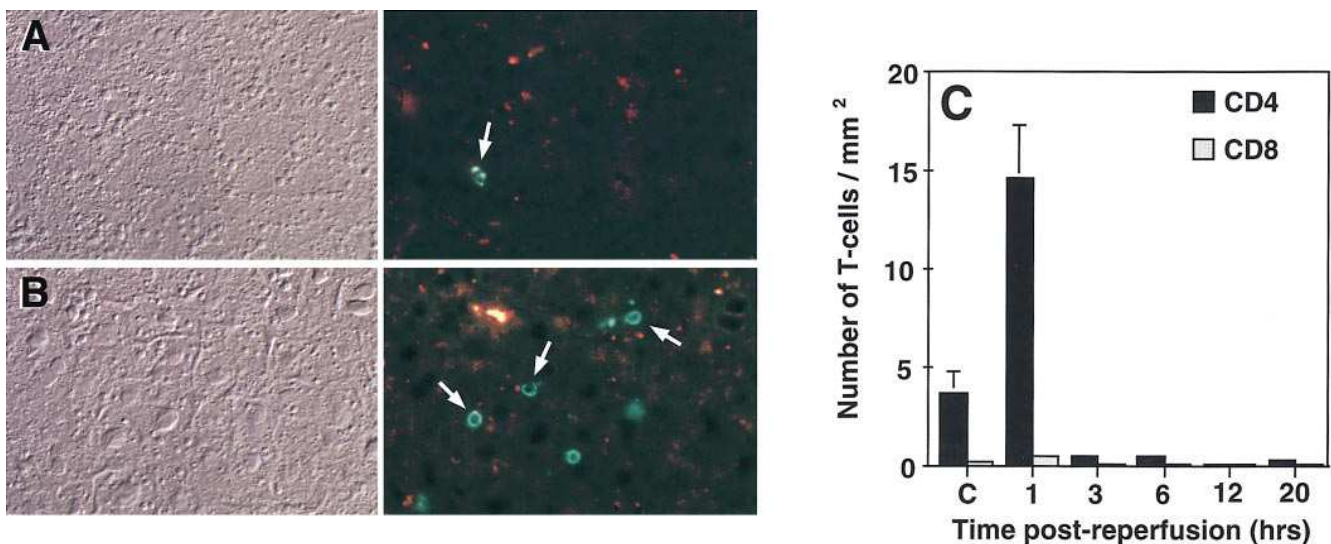


Figure 5. T-lymphocyte infiltration in the liver after I/R-induced injury. The kinetics of T-lymphocyte infiltration in the liver after 90 min of ischemia and 1, 3, 6, 12, and 20 h of reperfusion was examined by immunofluorescent staining using anti-mouse CD4 and CD8 FITC antibodies. The numbers of resident T cells were analyzed in nonischemic sham-operated control BALB/c mouse liver section from three independent mice. Total numbers of immunoreactive cells were quantitated in three nonserial sections of the ischemic lobes from three independent animals for each reperfusion time point. A is a representative image of a liver section taken from a control animal, while B represents an animal that underwent 90 min of ischemia and 1 h of reperfusion. Nomarski and fluorescent photomicrographs are shown on the left and right of each panel, respectively. All photomicrographs were taken with a 40 \times objective and arrows indicate immunoreactive CD4⁺ T-lymphocytes. C summarizes morphometric analysis quantifying the number of CD4⁺ and CD8⁺ T cells/mm² seen in control and 90-min ischemic livers that underwent 1, 3, 6, 12, and 20 h of reperfusion. Sham-operated control mice that were not subject to I/R injury are marked C. The number of immunoreactive CD4⁺ T cells within ischemic lobes increases fivefold after 1 h of reperfusion as compared with sham-operated animals (Student's *t* test, $P < 0.05$). No significant differences were seen in the extent of CD8⁺ T cell infiltration at any time points.

animals (8.5 ± 2.3 cells/field) within ischemic lobes at 20 h of reperfusion (Fig. 4 C). These results substantiate findings with adoptive transfer of T cells in nu/nu animals and provide more definitive evidence that CD4⁺ but not CD8⁺ T cell subsets are important mediators of inflammatory responses associated with subacute liver injury. To mechanistically determine when T cells may act to mediate the post-I/R subacute inflammatory response, we performed kinetic studies aimed at elucidating the time course of T-lymphocyte recruitment to the ischemic lobes of the liver after reperfusion.

Kinetics of T cell infiltration into the ischemic liver. To evaluate the temporal relationship of T cell recruitment within the inflammatory cascade after I/R in the liver, we followed the time course of T cell infiltration in postischemic and reperfused livers from BALB/c mice. To this end, we compared the number of CD4⁺ and CD8⁺ T cells in sham operated animals with mice that underwent 90 min of ischemia and 1, 3, 6, 12, and 20 h of reperfusion. Results depicted in Fig. 5, A and B demonstrate maximal fivefold increase in the infiltration of CD4⁺ T cells to ischemic lobes at 1 h postreperfusion, which decreased more than 10-fold by 3 h of reperfusion. No significant change in the extent of CD4⁺ T cell infiltration was observed at later time points of 6, 12, and 20 h postreperfusion. However, T cells were usually not found in necrotic areas of the liver, and may in part account for the decrease in total T cells/mm² at the later time points. In summary, transient CD4⁺ T cell infiltration to ischemic lobes occurred very early within the process of reperfusion and suggests that this cell type may mediate early events that activate the subacute inflammatory cascade. In contrast, no changes in the number of CD8⁺ T cells recruited to the liver were seen throughout the time course of reperfusion after 90 min of ischemia and remained at endogenous levels seen in control mouse livers (Fig. 5 A). These findings suggest that CD4⁺ T cells are likely early cellular mediators of I/R inflammatory responses.

Discussion

The mechanisms of ischemic damage in the liver associated with reoxygenation is of great clinical relevance in understanding and preventing early graft failure in orthotopic liver transplants. In addition to acute free radical-induced injury, subacute inflammatory responses play a major role in post-I/R organ injury, which leads to a decline of liver function and potentially increased organ immunogenicity leading to graft rejection. In the present study, the analysis of genetically defined T cell-deficient mice has provided a unique animal model for studying the immunology of subacute inflammatory responses after I/R in the liver. Our findings, which demonstrate a substantially reduced subacute inflammatory-mediated I/R injury response in nu/nu mice, as compared with T cell-competent BALB/c mice, suggest that T-lymphocytes are required for complete activation of post-I/R inflammatory responses. Three pathophysiologic indexes including serum GPT levels, percent hepatocellular necrosis, and neutrophil infiltration were all reduced in nu/nu mice as compared with immune competent BALB/c mice. Furthermore, the involvement of T cells in mediating subacute I/R inflammatory injury was supported by adoptive transfer experiments of T cells into nu/nu mice, which were capable of increasing both the subacute serum GPT levels (20 h) and neutrophil influx to damaged tissue.

Studies aimed at elucidating whether Tc and/or Th T cell subsets were involved in mediating this subacute inflammatory response used in vivo depletion of CD8⁺ or CD4⁺ T-lymphocytes in immune-competent BALB/c mice. Results from these experiments demonstrated that CD4⁺ but not CD8⁺ T-lymphocyte depletion was capable of reducing both the extent of injury and neutrophil infiltration during the subacute phase of I/R liver damage. These findings suggest that CD4⁺ T cells are a predominant mediator of the I/R-induced inflammatory responses. Additionally, the fact that CD4⁺ T cell influx to the liver after ischemia occurred within the first hour of reperfusion suggests that this cell type may mediate very proximal events that are responsible for activation of the subacute inflammatory cascade. In contrast, CD8⁺ T cell influx to the liver after I/R remained unchanged and suggests that this cell type does not play an important role in mediating postreperfusion inflammatory events in the liver after ischemia. In summary, these results suggest that in the absence of CD4⁺ T cells, the liver has an improved capacity to recover from oxidant-mediated damage. Furthermore, one might predict that therapeutic intervention directed at Th-lymphocyte activation pathways after I/R injury could yield substantial therapeutic benefits in transplantation through abrogation of subacute neutrophil inflammatory responses.

The mechanism(s) by which neutrophils are recruited after I/R liver injury is presently only partially understood. Previous studies have evaluated the increased adherent characteristics of neutrophils to endothelium in the liver after I/R. To this end, studies in ICAM-1-deficient transgenic mice have demonstrated the importance of this adhesion molecule in the recruitment of neutrophils in a model of LPS-induced liver damage (34). These studies demonstrated a protection against leukocytosis in the livers of ICAM-1-deficient mice treated with LPS. Furthermore, the mechanism underlying this protection was proposed to be distal to the inciting events that promote TNF α and IL-1 cytokine production, since these cytokines remained elevated in both wild-type and ICAM-1-deficient mice after LPS injury. Similarities between this toxic shock model of liver damage and that encountered in I/R-induced liver injury include the production of TNF α and IL-1 (11–13). Such hepatic cytokines have been suggested to be produced by Kupffer cells within the early phases of ischemic liver injury before reperfusion and have been implicated in amplification of subsequent neutrophil inflammatory responses (11, 12). These cytokines may have a direct role in activation of neutrophils or in contrast may activate other hematopoietic cell types (e.g., T cells) involved in regulating the injury responses to I/R in the liver. Findings from the present study have provided new insights into the potential involvement of T cell lineages in the recruitment and amplification of neutrophils to the damaged liver after I/R. Specifically, we propose that CD4⁺ T-lymphocytes may be important mediators of these responses.

CD4⁺ T cells can be subdivided into at least two subpopulations, Th1 and Th2. Th1 cells, which secrete IFN γ , TNF β , and GM-CSF (known to have stimulatory effects on Kupffer cells and neutrophils), may represent the best candidates for mediating this process of inflammation (30). Of these various T cell-secreted cytokines, IFN γ and TNF β are known to be potent activators of Kupffer cells and may likely promote local secretion of TNF α and IL-1, which in turn act to facilitate changes in endothelial adherence characteristics of neutrophils through either direct activation of neutrophils themselves or

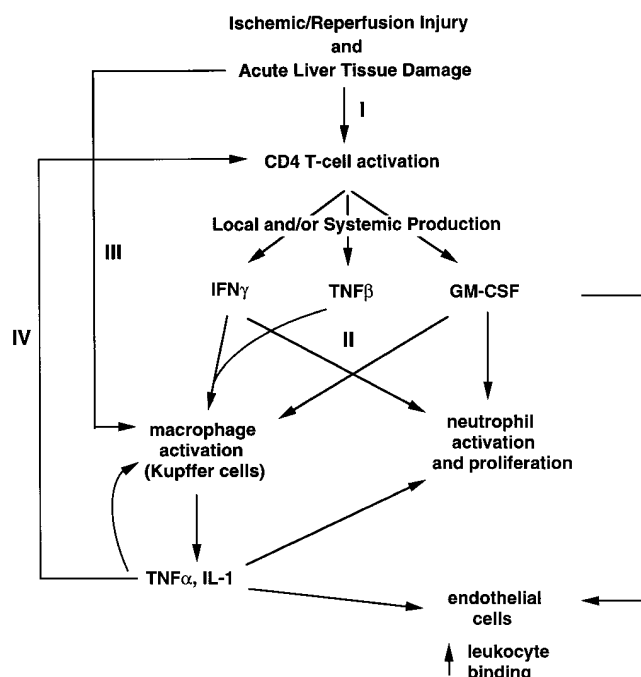


Figure 6. Potential mechanism(s) of T-lymphocytes activated inflammatory response after I/R injury in the liver. This schematic proposes potential mechanism(s) by which CD4⁺ T-lymphocytes mediate the activation of neutrophil inflammatory responses after ischemia and reperfusion in the liver. One potential hypothesis suggests that the stimulus of ischemia/reperfusion injury triggers activation directly in resident CD4⁺ T cells (pathway I). Once activated, CD4⁺ T cells may secrete a number of cytokines including IFN γ , TNF β , and GM-CSF, which either directly or indirectly (through Kupffer cell-secreted cytokines) activate neutrophils to infiltrate the liver (pathway II). Alternatively, the I/R stimulus may directly activate resident Kupffer cells within the liver (pathway III), which in turn activate circulating CD4⁺ T cells through secreted cytokines (pathway IV). Despite the present speculation as to the exact cytokine pathways by which CD4⁺ T cells interact within the amplification of neutrophil inflammatory responses after I/R injury, the significantly reduced subacute phase I/R injury in the absence of T cells demonstrates that some component of T cell activation is critical for this response.

indirectly through changes in surface adhesion molecules on endothelial cells. Furthermore, Th1-secreted IFN γ and GM-CSF may also act directly on neutrophils and enhance their partitioning to damaged liver tissue.

The primary question at present is whether T cell involvement lies proximal or distal with respect to the activation of Kupffer cell cytokine secretion, which has been demonstrated to play a major role in neutrophil recruitment. If T cell involvement lies proximal to the activation of Kupffer cells, this would imply that circulating T cells within the liver are directly activated by ischemia and reperfusion (i.e., changes in the cellular or extracellular redox state) and are potentially involved in initiating Kupffer cellular responses. Alternatively, T cells may be critical in amplifying primary Kupffer cell cytokine responses within the initial phases of injury. This hypothesis is attractive given the fact that secretion of TNF α by Kupffer cells has been suggested to occur before reperfusion. However, since CD4⁺ T cells are also resident in the liver before

reperfusion, it is impossible to rule out primary inciting activation of Kupffer cells by CD4⁺ T cells during the ischemic period. In addition, T cell-mediated effects could be amplified by other ectopic sites such as the spleen. In support of this hypothesis, a recent paper by Okuaki et al. (35) has demonstrated reduced neutrophil infiltration and attenuation of I/R injury in animals subjected to splenectomy as compared with controls. In the context of this present study, these data suggest that T cells at distant sites to the liver (e.g., the spleen) may also be involved in the inflammatory process. This action could occur by recruitment of T cells to the liver or alternatively through soluble mediators such as cytokines. Regardless of the exact mechanisms of action, since activation of CD4⁺ T cell recruitment to the liver occurs very early within the first hour of reperfusion, one would anticipate that T cells play a critical early role in amplifying the post-I/R inflammatory cascade. Fig. 6 summarizes potential mechanisms by which CD4⁺ T-lymphocytes may mediate a subacute inflammatory response in the liver after I/R injury.

Activation of naive T cells has traditionally been demonstrated to require recognition of foreign antigens bound to a self-MHC molecule together with costimulatory signals by an antigen presenting cell. However, in I/R injury, the lack of available foreign antigen's for presentation by antigen presenting cells suggests that activation of T-lymphocytes after I/R injury may occur via an antigen-independent pathway. Alternatively, self-antigens may be modified by generated reactive oxygen species so they appear foreign to T-lymphocytes. An antigen-independent activation mechanism of T-lymphocytes has recently been described by Bacon et al. (36) that involves the chemokine Rantes. Additionally, protein modifications due to oxidation with associated inflammatory responses have been postulated for cases of liver cirrhosis (37).

Despite the present lack of a defined cytokine-mediated pathway, these studies conclusively implicate T-lymphocytes as key mediators in the pathoprogession of I/R inflammatory responses in the liver. Elucidation of the cytokine pathways for T-lymphocyte activation of neutrophil recruitment in the liver after I/R may ultimately yield clinically relevant modalities for the amelioration of acute rejection in orthotopic liver transplants. Furthermore, since the major T cell subsets involved in graft rejection may be different than those involved in subacute phase post-I/R inflammatory responses (i.e., Tc [CD8⁺] versus Th [CD4⁺] cells, respectively), the rational design of effective therapies to prevent graft rejection necessitates the need for a concrete understanding of the cellular and cytokine pathways involved in these two pathophysiologically unique T cell-mediated responses. Neutrophil-mediated damage secondary to I/R injury is a pathophysiologic process seen in a number of additional organs including the heart, brain, lung, and kidney. Studies aimed at determining the T cell dependency of these post-I/R neutrophil responses, as seen in the liver, could lead to potential novel anti-T cell-directed therapies for other types of ischemically induced injuries as seen in myocardial infarction and stroke.

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